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# Synthesis and characterization of a lubricin mimic (mLub) to reduce friction on the articular cartilage surface

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By Alexandra M. Lawrence

Entitled

Synthesis and Characterization of a Lubricin Mimic (mLub) to Reduce Friction on the Articular Cartilage Surface

For the degree of Master of Science in Biomedical Engineering

Is approved by the final examining committee:

Alyssa Panitch

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Corey Neu

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Sarah Calve

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Approved by Major Professor(s): Alyssa Panitch and Corey Neu

Approved by: George Wodicka

Head of the Departmental Graduate Program

4/7/2015

Date



SYNTHESIS AND CHARACTERIZATION OF A LUBRICIN MIMIC (MLUB) TO  
REDUCE FRICTION ON THE ARTICULAR CARTILAGE SURFACE

A Thesis

Submitted to the Faculty

of

Purdue University

by

Alexandra M Lawrence

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science in Biomedical Engineering

May 2015

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West Lafayette, Indiana

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## ABSTRACT

Lawrence, Alexandra M. M.S.B.M.E., Purdue University, May 2015. Synthesis and Characterization of a Lubricin Mimic (mLub) to Reduce Friction of the Articular Cartilage Surface. Major Professors: Alyssa Panitch and Corey Neu.

The lubricating proteoglycan, lubricin, facilitates the remarkable low friction and wear properties of articular cartilage in the synovial joints of the body. Lubricin lines the joint surfaces and plays a protective role as a boundary lubricant in sliding contact; down-regulation of lubricin is associated with cartilage degradation and the pathogenesis of osteoarthritis. An unmet need for early osteoarthritis treatment is the development of therapeutic molecules that mimic lubricin function and yet are also resistant to enzymatic degradation common in the damaged joint. Here, we engineered a lubricin mimic (mLub) that resists enzymatic degradation and binds to the articular surface to reduce friction. mLub was synthesized using a mucin-like chondroitin sulfate backbone with collagen II and hyaluronic acid (HA) binding peptides to promote interaction with the articular surface and synovial fluid constituents. In vitro and in vivo characterization confirmed the binding ability of mLub to isolated collagen II and HA, and to the cartilage surface. Following trypsin treatment to the cartilage surface, application of mLub, in combination with purified or commercially available hyaluronan, reduced the coefficient of friction to control levels as assessed over macro- to micro-scales by rheometry and atomic force microscopy. In vivo studies demonstrate an mLub residency time of less than 1 week. Enhanced lubrication by mLub reduces surface friction, ideal to help suppress the progression of degradation and cartilage loss in the joint. mLub therefore shows potential for viscosupplementation treatment in early osteoarthritis following injury.



## CHAPTER 1. INTRODUCTION

Movement in animals is made possible in part by joints of the musculoskeletal system. Synovial joints, including the knee and elbow, are characterized by articular cartilage providing smooth sliding surfaces between contacting bones allowing movement in daily life. Proper lubrication is needed at the articular cartilage surface of these bones to ensure low friction and wear. Injury and failure of the articular cartilage leads to improper function of the load bearing and lubrication. This improper function leads to the symptoms of osteoarthritis<sup>1</sup>.

Osteoarthritis (OA) is a chronic joint disease characterized by the degradation of the extracellular matrix (ECM) in articular cartilage. This degradation is facilitated by several matrix metalloproteinases<sup>2</sup>. These enzymes break down ECM molecules including collagen II, HA, and aggrecan as well as molecules in the synovial fluid like HA and lubricin. Loss of lubricin in particular decreases the lubricating ability by the synovial fluid and increases friction and wear on the cartilage. The further wear on the cartilage leads to the symptoms of OA including painful and swollen joints. Down-regulation of the lubricin is associated with cartilage degradation and the pathogenesis of OA<sup>3</sup>.

Lubricin creates boundary lubrication, characterized as a molecular thin film between sliding surfaces in order to reduce friction and wear<sup>4</sup>. Animal models of joint injury have also shown treatment with lubricin has resulted in a reduction of cartilage degradation<sup>5</sup>. In addition, the friction coefficient of cartilage surface correlates with OA severity<sup>6</sup>. In the healthy joint, lubricin provides a chondroprotective layer against mechanical wear due to creating an adsorbed molecular layer that provides shock absorption<sup>7,8</sup>; this protective layer is disrupted in joint diseases like OA.

Lubricin, also commonly referred to as superficial zone protein (SZP) or proteoglycan 4 (PRG4), is a large glycoprotein found in the superficial zone of articular cartilage and the synovial fluid<sup>7,9</sup>. The molecule is encoded by the gene PRG4 and expressed by the cells in the surrounding synovial and cartilage tissue included chondrocytes, the cellular component of cartilage. The molecular weight of lubricin is approximately 227.5 kDa by sedimentation-equilibrium measurement<sup>10</sup>. Lubricin is composed of both proteins and glycosaminoglycans<sup>9</sup>. With multiple domains, the molecule expresses multiple biological functions<sup>9,11</sup>. The glycoprotein contains a somatomedin B (SMB) and a hemopexin-like (PEX) domain<sup>2,11</sup>. These domains suggest the functions like regulating the complement and coagulation systems, mediating extracellular matrix attachment, and promoting cell attachment and proliferation<sup>2,11</sup>. The hemopexin-like domain has been shown to interact with hyaluronic acid (HA)<sup>2,11,12</sup>. Lubricin attachment to the articular surface of the cartilage may be upheld by disulphide bond formation<sup>9</sup>. Between the N-terminal SMB domain and the C-terminal PEX domain is a mucin-like domain, which is available for O-linked glycosylation. This domain carries several negatively charged sugars, including chondroitin sulfate, that create a large water-holding capacity to contribute to boundary lubrication<sup>11</sup>. Lubricin plays a critical role in boundary lubrication at the articular surface and reduces friction<sup>9,11,13,14</sup>. The concentration of lubricin in the synovial fluid is on the order of 200 µg/ml<sup>15</sup>. Studies have shown that hyaluronic acid (HA), another component of articular cartilage and synovial fluid, interacts with lubricin to reduce friction<sup>13,16</sup>. Exposure of the synoviocytes and chondrocytes to cytokines and growth factors cause cells to up or down regulate the release of lubricin. Proinflammatory interleukin-6 (IL-6) has been shown to reduce the expression of secreted lubricin while transforming growth factor-β (TGF-β) has been shown to upregulate lubricin synthesis<sup>17,18</sup>. Due to its role in reducing friction, it is not surprising that mechanical stimuli also play a role in lubricin secretion. Studies have looked at the pathogenesis of osteoarthritis and observed a decrease in the expression of PRG4 by chondrocytes<sup>3</sup>. On the other hand, other studies have shown injurious compression to the cartilage has led to an increase of PRG4 expression and secretion in the superficial zone in later stages<sup>19</sup>. Studies have also shown a higher expression of PRG4 in the anterior regions of the joint, or the load bearing

regions where there is more shear stress<sup>6,20</sup>. Taken together, these studies suggest that there exists both biochemical and mechanical regulation of lubricin gene expression and protein secretion.

Currently, the disease-modifying treatment for OA is limited but some work has started looking into supplemental lubricant injections into the synovial fluid. One approach involves injecting purified human lubricin and/or hyaluronic acid into the synovial fluid to reduce cartilage damage<sup>21</sup>. Other researchers have looked into synthetic lubricating solution to decrease friction and protect the cartilage<sup>8</sup>. Currently on the market, Synvisc is a 0.8 % high molecular weight HA formulation injected into the synovial fluid, claiming OA pain relief for 6 months. There are varying opinions as to the efficacy of Synvisc, which perhaps results from its susceptibility to enzyme degradation and a low residency time<sup>8</sup>. Instead, there is a need to a mimetic molecule that will maintain the function of a lubricating molecule but not the structure.

Our solution is use a proteoglycan mimic of lubricin (mLub) to imitate the lubricating properties of lubricin but without mimicking the structure. We have synthesized a molecule containing collagen II and HA binding peptides. The actual binding to collagen II and HA as well as ex vivo cartilage has been confirmed. We have also optimized the formulation of the molecule for best results along the way. We have confirmed the lubricating function of mLub by measuring friction on cartilage macroscopically and microscopically. Lastly, we have started in vivo testing for residency time of mLub on the articular cartilage.

## CHAPTER 2. SYNTHESIS AND CHARACTERIZATION OF MLUB

We have developed a lubricin mimic (mLub) to possess several characteristics for success in lowering the friction of cartilage in osteoarthritis. The basis of the molecule formulation originated in our lab with an aggrecan mimic<sup>22</sup> which contains a chondroitin sulfate (CS) backbone with ~20 covalently conjugated hyaluronic acid (HA) binding peptides (Fig. 1a). We noted that the aggrecan mimic diffused into damaged cartilage and protected the cartilage in an osteoarthritic environment<sup>23</sup>. However, in unpublished work with a collagen type II binding variant of the aggrecan mimic we found that the molecule collected at the cartilage surface rather than diffusing in. This led to the development of the lubricin mimic described herein. The CS backbone, a molecule already found on lubricin and other cartilage proteoglycans, provides the molecule with the important negative charges found on lubricating molecules. The addition of collagen II binding peptides allows the molecule to bind to the collagen II on the surface of the cartilage. Next, adding HA binding peptide provides the mimic to bind with HA on the cartilage surface as well as in the synovial fluid to further reduce friction, another characteristic of lubricin. These two binding peptides allow the mimic to create a lubricating boundary on the cartilage surface (Fig. 1b.) This molecule will mimic the function but not the structure of lubricin, which may prevent susceptibility to enzymatic degradation, unlike native lubricin present in the cartilage and synovial fluid during in OA. We originally started with adding 10 moles of each HA and collagen II binding peptides per mole of CS as a start. After brief friction data collection (Fig. 2), we changed the molecule to possess 15 moles of collagen II binding and 5 moles of HA binding peptides per mole of CS and found better results, as shown later in this paper. We estimated the molecular weight of the mLub15 to be 107 kDa by summing the known molecular weights of the

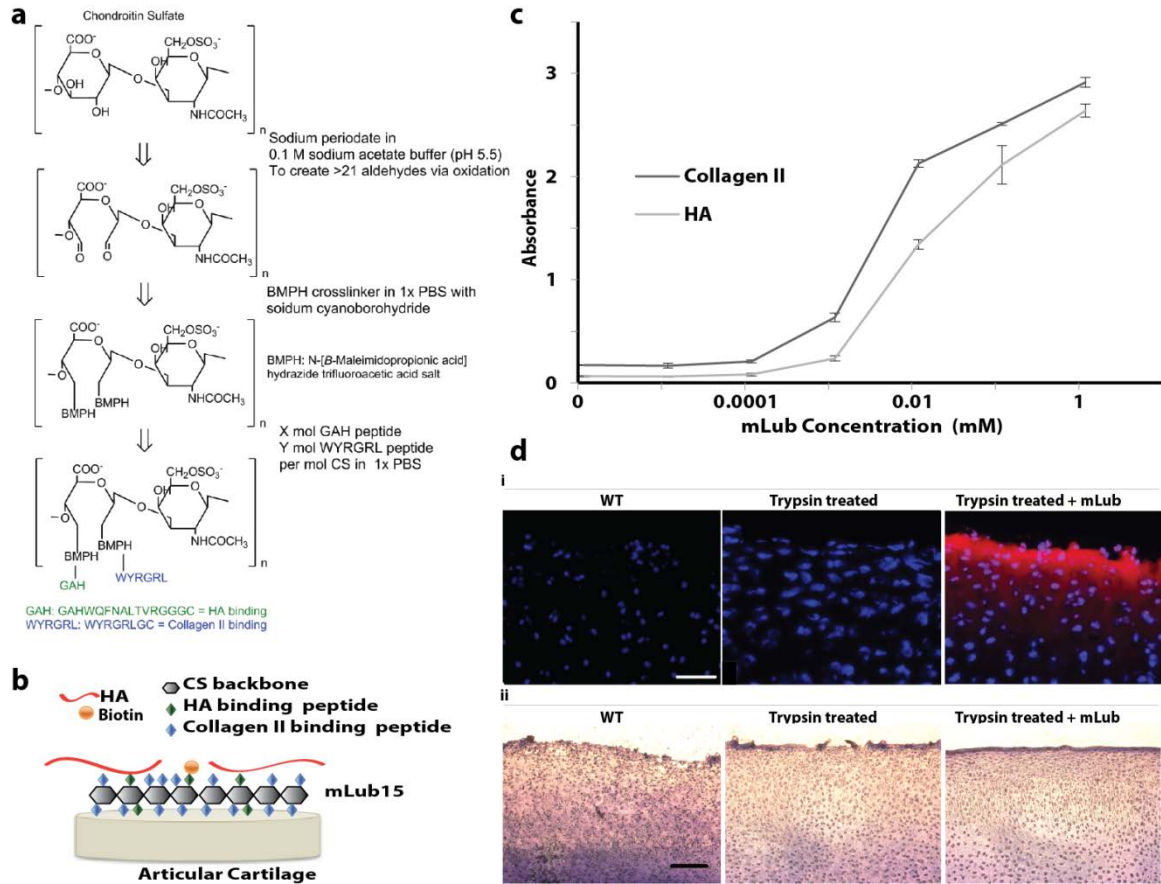
components of the molecule. The molecular weight is half of native lubricin and yet we see lubrication (Fig. 2).

After synthesizing mLub, its ability to bind both HA and collagen type II was validated (Fig. 1c). HA and collagen II were coated onto well plates and varying mLub dilutions were incubated in the coated wells. In order to probe for the mLub, on average one biotinylated-HA-binding peptide per CS backbone was conjugated during mLub synthesis. Bound mLub was then probed with streptavidin-fluorophore and the resultant absorbance correlated with mLub binding. Using this method we confirmed that both the binding peptides are functional on mLub. The data also shows that HA binding is weaker than collagen II binding, which is expected given the greater number of collagen II binding peptides than HA binding peptides on the mLub molecule.

To evaluate the binding of mLub to cartilage and to evaluate the ability of mLub to reduce friction at the cartilage surface, an ex vivo bovine cartilage model was used. Articular cartilage from the load bearing regions of bovine knee joints was harvested and used for all ex vivo studies described herein. To mimic osteoarthritis, cartilage plugs were subjected to 3 hours incubation with trypsin to deplete glycosaminoglycan content in the cartilage. This was confirmed with a Toluidine blue stain (Fig. 1dii). Toluidine blue binds to GAGs thus reduced staining is an indication of GAG removal. The untrypsinized WT cartilage sample staining is more saturated throughout the tissue compared to the two samples treated with trypsin. The lack of proteoglycan replenishment was confirmed using the toluidine treatment. Next, we confirmed mLub binding to the surface. A solution of mLub was added to the surface of cartilage plugs and rinsed before cutting 10  $\mu$ m cryosections. The sections were then stained by fluorescent streptavidin to probe for bound mLub (Fig. 1di). The clear presence of red streptavidin on the images versus no staining on the WT or trypsin treated alone cartilage proves that the mLub is binding to the cartilage surface and supports investigation of the lubricating function. It is important to note that the trypsin solutions were dissolved in HBSS plus a protease inhibitor cocktail (PIC). One of the inhibitors is known to inhibit trypsin yet we see a difference between WT and trypsin treated plugs. After treatments

with trypsin, the solution the cartilage plug sits in is observed to be cloudy compared to the solution of the plugs with no trypsin, showing the presence of degraded proteoglycans even with the PIC. With evidence from the toluidine blue staining, friction differences and physical differences between WT and Trypsin, we believe that the PIC only had minimal effects on inhibiting trypsin.

We have also designed this molecule to resist enzyme degradation from hyaluronidase, MMPs or aggrecanase unlike native molecules in the cartilage and synovial fluid during osteoarthritis. We have developed a lubricin mimic (mLub) to possess several characteristics for success in lowering the friction of cartilage in osteoarthritis. We have designed this molecule to lack the enzymatic degradation targets which has been done and evidence has been shown for previously. Further, like the aggrecan mimic, we will investigate the ability of mLub to protect other molecules found in the cartilage and synovial fluid from enzymatic degradation.



**Figure 1: Synthesis of biomimetic lubricin molecule shown to bind to collagen II and HA and spatially localize on cartilage surface.** a, Reaction schematic for the synthesis of mLubX with the addition of HA and collagen II binding peptides to a chondroitin sulfate backbone. b, Schematic of mLub15 binding to the articular surface and HA in the synovial fluid. c, mLub15 binding curves with HA and collagen II on coated well plates. d, mLub15 binding on cartilage cryosections i) cartilage labeled with DAPI for cell nuclei (blue) and streptavidin for biotinylated mLub (red). ii) Toluidine blue stained cartilage cryosections to show proteoglycan depletion by trypsin. White scale bar = 50um, black scale bar = 200um.

### CHAPTER 3. MLUB REDUCES COEFFICIENT OF FRICTION AT THE MACROSCALE

To test the lubrication properties of the mLub, coefficient of friction (COF) on cartilage was first studied on a macroscopic scale. A rheometer was used to measure torque and normal force under constant load in order to calculate the COF (Fig. 2a). The static friction between the WT cartilage and the trypsin treated cartilage were significantly different, with trypsin treated cartilage having a higher measured friction. No improvement in friction over trypsin treated was seen following mLub10 (10 collagen binding peptides per CS) alone, mixed with or followed by a treatment of Synvisc (clinical inject of an HA solution). Because these treatments were able to fully restore the COF to WT values a different mLub formulation with fewer HA-binding peptides and more collagen type II-binding peptides was explored. Next, treatments of mLub15 (15 collagen II binding peptides) alone or followed by Synvisc were explored. Each treatment group had a different n value (ranging from n=9 to 12). Differences in sample numbers (n) between groups are due to difficulty in maintaining the adhesion of the cartilage plug to the rheometer geometry head. If after raising the geometry head after a run it was observed that the plug separated from the geometry, then the data was thrown out due to the uncertainty of proper torque measurements. Statistical significance is marked,  $p=0.001$  for static COF and  $p=0.011$  for kinetic COF.

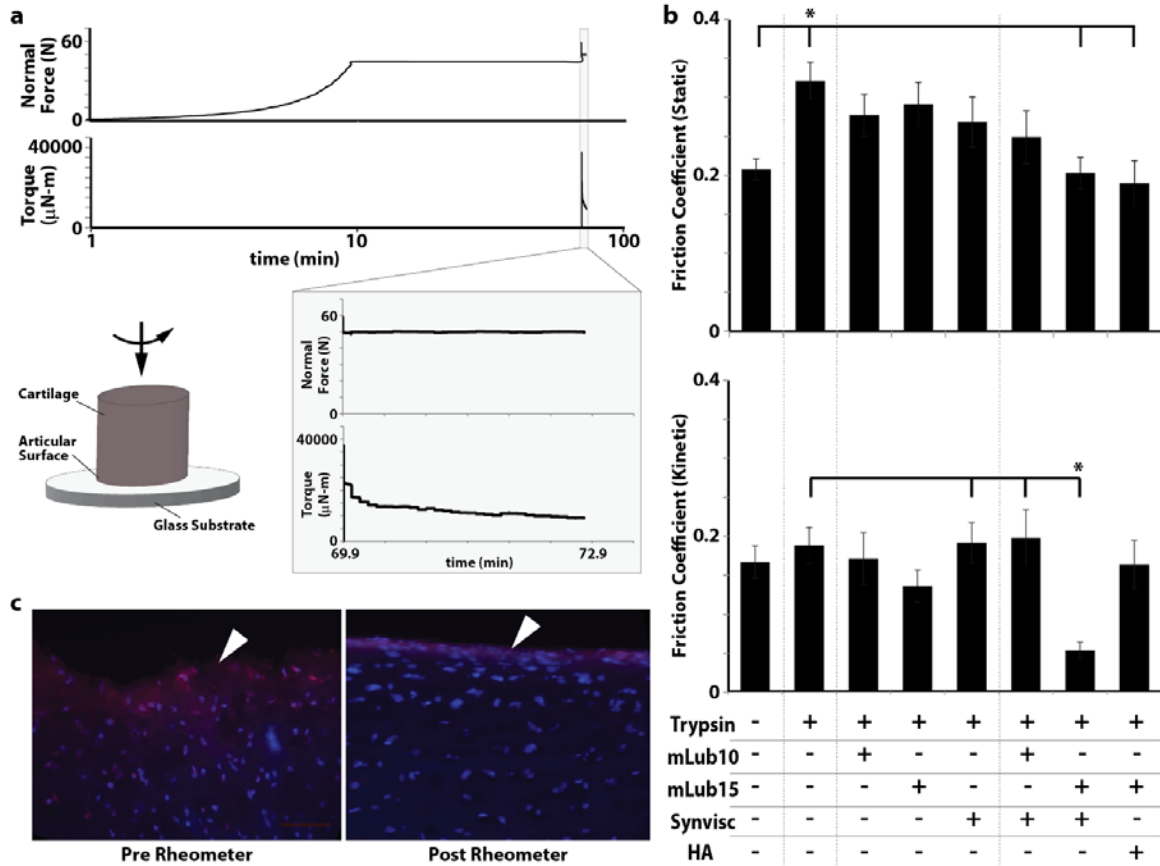
The static coefficient of friction on cartilage was restored after treatments of mLub15, rinse, and then Synvisc. Hence, the remaining studies were done only with mLub15 and not mLub10. A treatment of mLub15 followed by HA (instead of Synvisc) was also investigated to model mLub15 interaction with HA present in the native synovial fluid. We found that this treatment also restored the coefficient of friction on the cartilage (Fig. 2b). Static COF represents the friction opposing impending movement while kinetic (or



dynamic) COF represents the friction opposing continual movement; static is usually larger than kinetic<sup>24,25</sup>. Both of these values may be important to investigate while looking at joint movement because people undergo both impending movement and continual movement. We see more changes within the groups when looking at static friction versus kinetic friction and this may be because adhesion plays a larger role in static friction while surface roughness plays the larger role in kinetic friction. The data in Fig. 2b confirms the ability of mLub to lower friction, which motivated us to look deeper into a microscopic scale (Fig. 3).

To confirm that the mLub remained bound to the cartilage surface following the compression and shear stress applied to the cartilage (similar to that seen during normal activity in vivo) fluorescent streptavidin staining was applied to cartilage samples cryosectioned following friction evaluation. There was a difference in observed staining demonstrating that mLub remained bound to the surface (Fig. 2c) after the testing. No comparison can be made based on staining observed before and after friction testing due to the morphology change from the compression of the cartilage sample during testing. This motivates further investigation of mLub residency time in vivo (Fig. 4)

Other studies have shown that briefly releasing the cartilage from loading enhances the lubrication on the cartilage surface<sup>26</sup>. Like in this study, the mLub has also shown an ability to replenish lubrication via lower friction and the fluorescent staining. Unlike this mentioned study, the cartilage was not released from the static load before slide and yet, lubrication was restored. Also, previously they have tested with natural molecules in the synovial fluid, while we tested with a synthetic molecule. With both natural and synthetic lubricant replenishment, lubrication can be restored. In another study, replenishment by lubricant reservoirs were investigated for friction on the cartilage surface<sup>4</sup>. Likely, the sliding of the cartilage on the surface creates reservoirs of attached mLub to reduce stress on the cartilage and lower friction



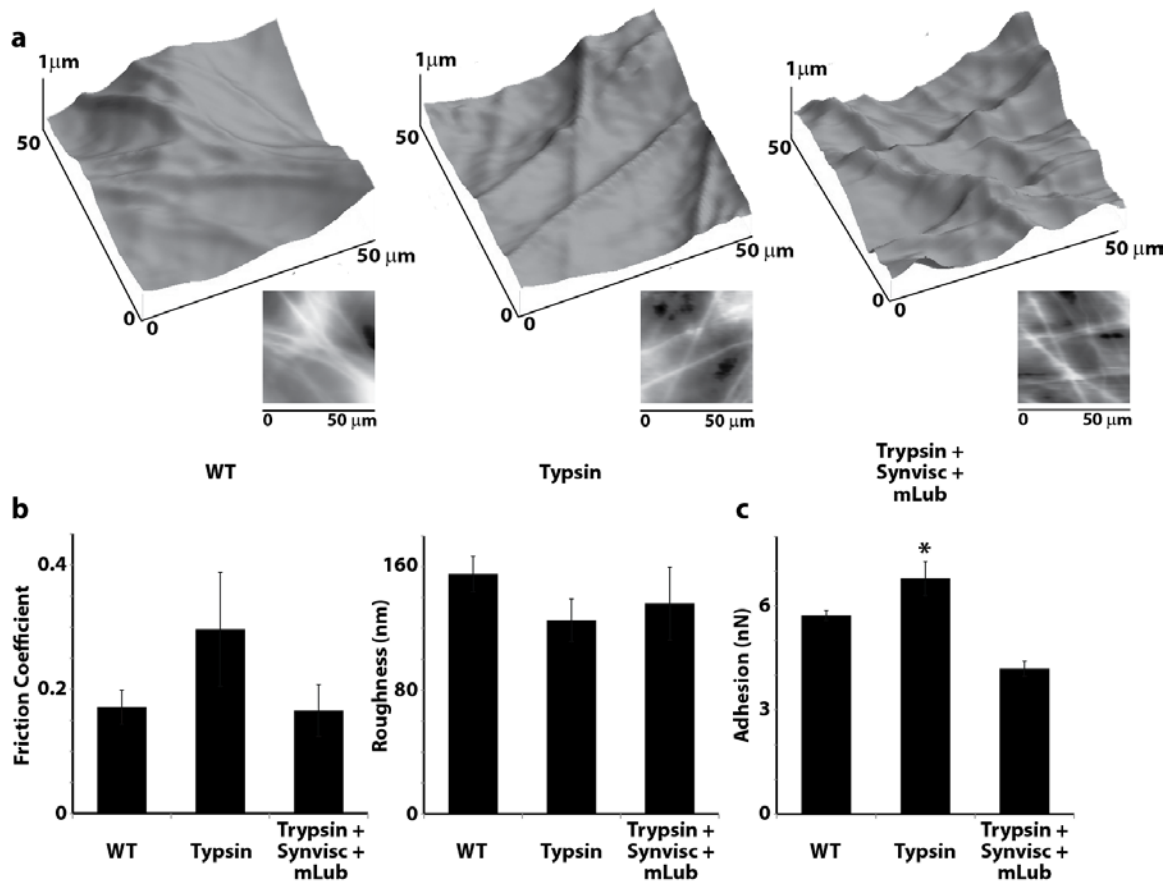
**Figure 2: Treatments of mLub15 and Synvisc or HA on cartilage restores the coefficient of friction following trypsin treatment.** a, Typical normal force and torque graphs during macro-scale coefficient of friction testing using a rheometer. b, calculated static and kinetic coefficient of friction values of each treatment group. In static COF, there is statistical difference ( $p < 0.05$ ) between the trypsin treated plug and the WT, mLub15 + Synvisc, and mLub15 + HA treatments. In kinetic friction, there is statistical difference ( $p < 0.05$ ) between the mLub15 + Synvisc treatment and the trypsin, Synvisc, and mLub10 + Synvisc treatments. Standard error bars are shown. c, Fluorescent staining images of cryosections of cartilage with mLub probed with streptavidin (red) and DAPI for nuclei (blue). The left image represents a cartilage sample that did not go through the compression and shear movements on the rheometer while the right image was cryo-sectioned after the rheometer test. There is still mLub present after compression and shear movement. White arrows point to the mLub covered cartilage surface.

#### CHAPTER 4. MLUB REDUCES COEFFICIENT OF FRICTION AND ADHESION, BUT NOT ROUGHNESS, AT THE MICROSCALE

After macroscale friction measurements were taken, microscale surface features were investigated using atomic force microscopy. A colloidal probe was used for contact AFM to take lateral force measurements. Since a significant trend was observed for macroscale friction between WT, trypsin, and trypsin plus mLub and Synvisc treatments, only these three groups were investigated further in the microscale. While measuring lateral force, a topography map is created (Fig. 4a) and cartilage fibers can be perceived. Coefficient of friction was calculated by dividing the lateral (or friction) force by the normal load of the AFM probe. A COF trend matching the macroscale data is observed without statistical significance ( $p = 0.29$ ) (Fig. 3b). No trend between the sample groups was observed for roughness ( $p = 0.39$ ) (Fig. 3b). Although adhesion data was collected on only a small subset of the samples ( $n=2$ ) adhesion forces measured on the trypsin-treated cartilage were significantly greater than those measured on the WT and mLub + Synvisc treated samples ( $p < 0.05$ ) (Fig. 3c). There was a lot of variance in measurements even between samples of the same treatment group so there is a possibility that a larger  $n$  value is needed to get stronger results.

The microscale friction properties may start to explain the macroscale friction data. Although insignificant, the trends match for COF measured. Since the macroscale data is statistically significant we can see that the mLub plus Synvisc treatment is more effective on a larger scale. The lack of difference between roughness values from the AFM may link with the lack of difference between measure kinetic friction WT and trypsin treated cartilage (Fig. 2b). Although it does not explain as well the significant difference between the trypsin alone cartilage with the trypsin and mLub plus Synvisc treated cartilage kinetic COF. With a stronger data set for adhesion measurements, a good argument for a

difference in adhesion to be projecting the differences in macroscale static COF could be made. Other studies have shown that adhesion and friction measured on the nanoscale with the AFM are related while roughness do not predict the adhesion and friction values on the nanoscale cartilage surface<sup>27</sup>. This may be why we see differences in friction and adhesion but not in roughness between the samples. Another reason why we see differences in the friction measurements between the macro- and micro- scale may be because of compounding fluid pressurization, as suggested in other studies. The COF measured by AFM represents the frictional response in the absence of interstitial fluid pressurization and may give different insights about the lubrication than macroscale measurements<sup>28</sup>.

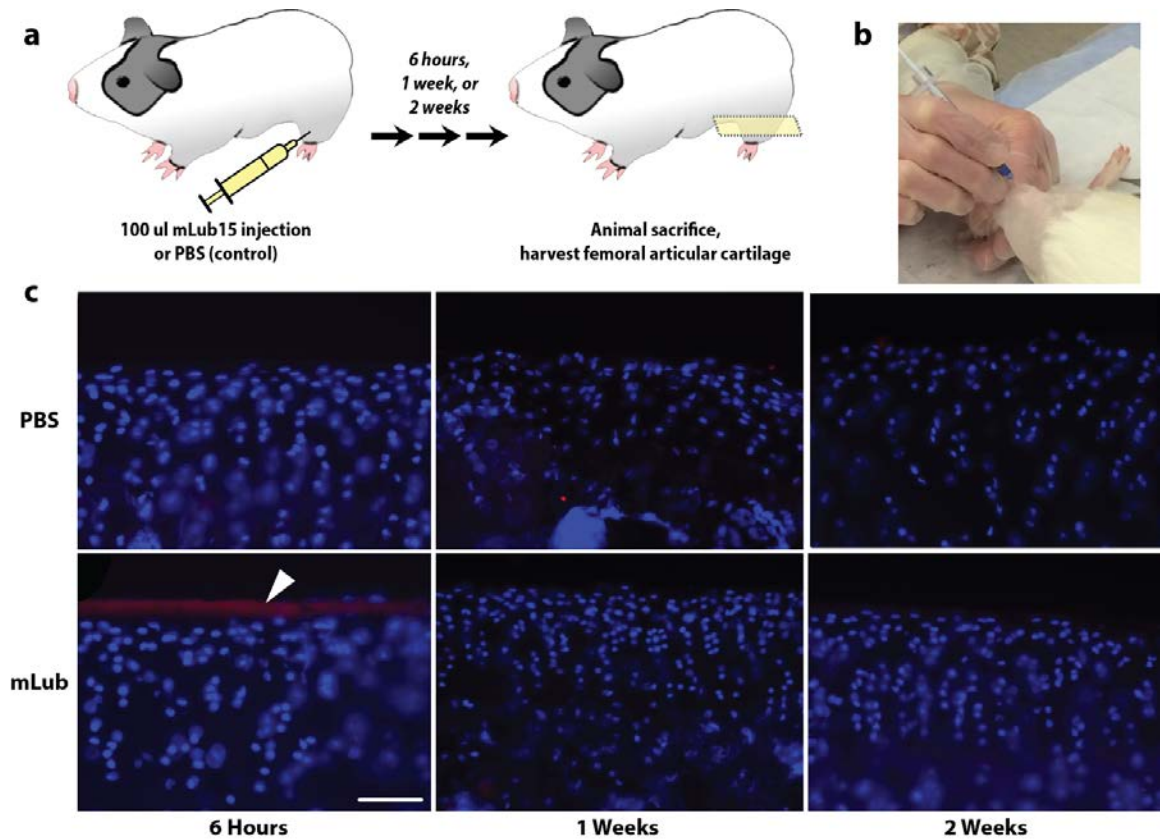


**Figure 3: Cartilage surface properties measured by atomic force microscopy.** a, 3D and 2D topography images of cartilage surfaces, WT, Trypsin treated, and Trypsin treated later followed by a mLub then Synvisc treatment. Images represent samples with roughness values near the values in b. b, Friction coefficient and roughness values of selected areas of cartilage surfaces ( $n = 9$ ) ( $p > 0.05$ ) Standard error bars are shown. c, adhesion values of a small subset of cartilage samples ( $n=2$ ) ( $p < 0.05$ ). Standard error bars are shown.

## CHAPTER 5. MLUB RESIDENCY TIME IN VIVO IS LESS THAN ONE WEEK

Initial in vivo studies were done to ensure that direct injections of mLub into the synovial space would allow mLub to bind to the cartilage surface. Dunkin Hartley guinea pigs show signs of OA as early as four months, thus guinea pigs approximately four months of age were used for initial binding studies. Injections of mLub and PBS were done through the patella tendon, to mimic clinical injections (Fig. 4b). The guinea pigs were allowed to move freely afterwards and until they were sacrificed. After sacrifice the femoral condyles were harvested (Fig. 4a). Cryosections were made and stained with fluorescent streptavidin for presence of mLub on the surface of the cartilage and DAPI to identify cells within the cartilage. mLub was found on the cartilage surface in the guinea pigs sacrificed after 6 hours but not after 1 or 2 weeks following injection indicating that the residency time of mLub is less than 1 week. Future in vivo studies will be performed to further define the mLub residence time, and to evaluate the chondroprotective qualities of the mLub.

As mentioned in the introduction, the onset of osteoarthritis is correlated with a low expression of lubricin while after some time; lubricin expression is much higher than normal levels. Introducing the mLub molecule, even with a residency time less than a week, during when expression is low in the synovial fluid may still be advantageous. We also know that the boundary layer naturally replenishes itself<sup>4</sup> so there is a possibility for mLub to act as a place holder for the boundary layer



**Figure 4: In vivo spatial localization of mLub to the articular cartilage surface with residency time of less than 1 week.** a, Cartoon schematic of in vivo study. b, picture of injection through the patellar tendon and into the synovial fluid. c, fluorescent imaging of the cartilage of guinea pig joints injected with PBS (control) or mLub and harvested after 6 hours, 1 week or 2 weeks. Fluorescent streptavidin is bound to the biotinylated mLub (red) and DAPI is bound to cell nuclei (blue). The scale bar represents 50  $\mu\text{m}$ .

## CHAPTER 6. MATERIALS AND METHODS

### 6.1 Synthesis of Lubricin Mimic (mLub)

The synthesis methods for lubricin mimic were modified from previous work done in our lab<sup>22</sup>. Vicinal hydroxyl groups on a chondroitin sulfate (CS) were oxidized with sodium periodate to create aldehyde functional groups. Briefly, 20 mg/ml CS (Sigma-Aldrich, St. Louis, MO) was dissolved in a 0.1 M sodium acetate buffer. For oxidation, the sodium periodate (Thermo Scientific, Waltham, MA) was added to allow for 21 aldehyde groups to form and allowed to react away from light. Gel filtration chromatography using an ÄKTA Purifier FPLC (GE Healthcare, Piscataway, NJ) was used to stop the reaction and purify the oxidized CS as previously described<sup>22,23</sup>. A crosslinker, *N*-( $\beta$ -Maleimidopropionic acid) hydrazide, trifluoroacetic acid salt (BMPH) (Pierce, Rockford, IL) was then added in excess along with sodium cyanoborohydride and allowed to react. Excess BMPH was then removed using gel filtration chromatography. The CS-BMPH was frozen and lyophilized before conjugating the binding peptides. The CS-BMPH was finally functionalized by the addition of an HA-binding peptide, GAHWQFNALTVRGGGC (GAH), and a collagen II-binding peptide, WYRGRL (Genscript, Piscataway NJ). Initially, approximately 10 moles of each peptide was added to a single CS backbone (mLub10), later this ratio was changed to approximately 5 moles HA-binding and 15 moles collagen II-binding peptides per mole of CS (mLub15). One GAH mole was biotinylated for characterization. The molecule was purified again and then frozen and lyophilized for storage at -80°C. The estimated molecular weight of mLub(15) is around 107 kDa, this value was calculated by summing the known molecular weights of the individual components of the molecule.



## 6.2 Characterization of mLub Binding In Vitro

The binding of mLub to HA was determined using a simple binding assay. 96-well black with clear bottom Greiner well plates were coated with 50 µg/ml HA (hyaluronic acid sodium salt from *streptococcus equi*) (Sigma-Aldrich) in 50 mM sodium carbonate and incubated overnight. After each step the wells were rinsed with PBS + Tween. Wells were blocked with 1% BSA in PBS for 1 hour then rinsed. A 10:1 dilution of the lubricin mimic was made and the solutions were added to the wells and incubated at 37°C for 1 hour and then rinsed. Streptavidin-HRP (R&D Systems, Minneapolis, MN) was added to each well and incubated for 20 minutes. After rinsing, a color solution was added to each well and incubated away from light for 20 minutes. 2N sulfuric acid was added to each well and the absorbance was read on a plate reader (SpectraMax, Molecular Devices) at 450 and 540 nm.

The binding of mLub to collagen II was also determined using a similar protocol with the HA. The well plate was coated with a 0.5 mg/ml solution of collagen II from chicken sternal cartilage (Sigma-Aldrich) in 10 mM HCl and incubated overnight. The rest of the procedure is the same as that for HA.

## 6.3 Cartilage Harvesting and Treatments

Cartilage plugs with a 7 mm diameter were harvested with a cork borer from ~5-month-old bovine knee joints obtained 24 hours after slaughter (Dutch Valley Foods). Samples were taken from the anterior regions of the joint at the load bearing regions<sup>20</sup>. Samples were washed three times in Hank's Balanced Salt Solution (HBSS) (Life Technologies, Carlsbad, CA) with a protease inhibitor cocktail (PIC) (PMSF, EDTA, iodoacetamide, benzamidine hydrochloride hydrate (Sigma-Aldrich), penicillin-streptomycin (Cellgro)). To mimic osteoarthritis, randomly selected samples were treated with a 0.5% trypsin solution for 3 hours to deplete the cartilage of some peptidoglycans while others were left as WT samples<sup>29</sup>. The samples were then rinsed with HBSS/PIC and then washed with fetal bovine serum for 10 minutes to deactivate the trypsin. Samples were stored in the

HBSS/PIC in 4°C and tested within 24 hours of harvest. As mentioned before in chapter 2 we note that the PMSF in the PIC is a trypsin inhibitor yet we still have evidence that the trypsin is not being inhibited and can still degrade the proteoglycans.

The lubricin mimic was dissolved to a concentration of 0.5 mg/ml, which is on the higher end of the range of concentrations found in synovial fluid<sup>30</sup>. Synvisc (Genzyme, Ridgefield, NJ) was obtained and diluted to a concentration of 3 mg/ml to be a concentration closer to the actual HA concentration in synovial fluid. HA solutions were made at a concentration of 3 mg/ml. Cartilage samples that were previously treated with trypsin were treated right before testing took place. Treatments were added on the surface of the cartilage twice, five minutes apart before the samples were rinsed and tested. If the treatment group including mLub and Synvisc/HA, mLub was added like described above, the cartilage was rinsed and then the Synvisc/HA treatment added and rinsed.

#### 6.4 Characterization of mLub Binding to Articular Cartilage

After treatment and rinsing, cartilage plugs were frozen at -20°C in O.C.T compound (Tissue Tek) and sectioned at 10 µm thickness. Sections were allowed to dry in room temperature and stored at -20°C before being fixed in 4% paraformaldehyde and then briefly rinsed with PBS. For the detection of the biotin-labeled mLub, fluorescent streptavidin 555 (Life Technologies) was diluted 1:200 in a donkey serum, triton, and sodium azide solution. DAPI 461 was diluted 1:500 in the same solution to stain the nuclei. The sections were incubated 30 minutes in the dark. The sections were rinsed and mounted before they were imaged under a fluorescent microscope (Leica Microsystems). This protocol was also followed after the *in vivo* cartilage harvesting.

Cryosections were also stained with toluidine blue to show the proteoglycan depletion by the 3 hour trypsin treatment. After the samples were taken out of the -20°C storage, they were rehydrated with water briefly. The slides were stained with 0.04% Toluidine Blue (Sigma-Aldrich) solution in 0.1 M acetic acid buffer for 10 minutes. The slides were rinsed in water 3 times and then counterstained with 0.02% Fast Green FCF solution

(Sigma-Aldrich). The slides were rinsed again and then dehydrated with 100% ethanol before they were imaged with a light microscope

### 6.5 Macroscale Measurement of Coefficient of Friction

To measure the COF of the cartilage, procedures from Schmidt *et al*<sup>31</sup> were loosely followed. To create a more unified radius, the cartilage samples were made into annuli by cutting a 3 mm diameter hole in the middle of the 7 mm diameter plug, creating a 2.6 mm effective radius,  $R_{eff}$ . The cartilage annulus was super glued onto the center of a 20-mm flat rheometer geometry head (AR G2, TA Instruments). A cleaned glass microscope slide was taped to the bottom plate of the rheometer. The geometry head was lowered enough for the cartilage plug to barely touch the glass slide. HBSS/PIC was added to surround the cartilage plug to keep the plug from drying out. The rheometer software was set to compress the samples at a rate of 0.002 mm/second until a 50 N normal force was measured. The plug then stayed at equilibrium for 60 minutes. Next, the samples were rotated with an angular velocity of 0.08726 rad/sec for 2 minutes. Torque and normal force were measured with the software. Static coefficient of friction (COF) was calculated by taking the maximum torque during the first 10 degrees (~2sec) and normal force and applying this equation.

$$\mu = \frac{T}{R_{eff}N}$$

The kinetic COF was calculated by averaging the COF calculated from the second rotation.

## 6.6 Microscale Measurement of Coefficient of Friction, Roughness, and Adhesion

Microscale measurements of the COF were taken using contact atomic force microscopy (contact AFM) with PicoView software. Silicone AFM probes with 2  $\mu\text{m}$  diameter borosilicate spherical tips (Novascan Technologies, Ames, IA) were used with a nominal pre-calibrated spring constant of 0.6 N/m. A lateral force calibration constant was calculated using Varenberg's improved wedge calibration method<sup>32</sup> and a TGF11 silicon calibration grating. This calibration constant allowed for converting force values with units of Volts to Newtons.

The friction measurements were taken on 50 micron square sections of the cartilage sample at a speed of 50  $\mu\text{m}/\text{sec}$ . The area was scanned with a 5V normal load applied (around 120 nN). The normal load (V) was converted to normal force (N) by multiplying by the deflection sensitivity and spring constant. While observing the friction force plots during data analysis, areas of the surface where the controller was not detecting the surface were observed. In order to take these areas out of the data analysis we programmed data analysis code areas with the presence of fibers and no controller interference. These areas were then used for analysis. The average area taken from each sample area was 971 squared microns with a standard deviation of 280 squared microns. The friction voltage signal was averaged for both the trace and retrace scan and then the difference between the two divided by two. This value was then converted to friction force by multiplying by the calibration constant. Averaged friction force divided by normal force to calculate coefficient of friction<sup>27,28</sup>.

Using the same areas taken for friction measurements, surface RMS roughness was calculated using the topography data in the following equation.

$$R = \sqrt{\frac{1}{n} \sum_{i=1}^n z^2}$$

Adhesion was also measured on the cartilage surface with the AFM. Sixteen points were sampled per fifty micron square and the adhesions values were averaged.

### 6.7 In Vivo Residency Study

An *in vivo* study was performed to measure the residency time of mLub in the synovial fluid and on the articular cartilage. Dunkin Hartley guinea pigs were chosen as a model due to their development of spontaneous osteoarthritis. The animals were allowed to age to 4 months before testing. The patella tendon was located on the hind legs of the animals for injection. Sterile injections of 100  $\mu$ L of 0.5 mg/ml mLub in PBS were injected into the synovial fluid behind the patellar tendon. The contralateral knee was injected with 100  $\mu$ L PBS for a control. After 6 hours, 1 and 2 weeks, the animals were sacrificed and the femoral condyles were harvested and prepared in O.C.T. compound (Tissue-Tek) for cryosectioning. Before this study, a 50 mg/ml Coomassie Brilliant Blue G-250 (Sigma-Aldrich) solution was injected for visualization to confirm accurate delivery into the synovial fluid. 15  $\mu$ m cryosections were stained with the same protocol as stated previously.

## CHAPTER 7. CONCLUSION AND FUTURE WORK

The lubricating proteoglycan, lubricin, facilitates the remarkable low friction and wear properties of articular cartilage in the synovial joints of the body. Lubricin lines the joint surfaces and plays a protective role as a boundary lubricant in sliding contact; down-regulation of lubricin is associated with cartilage degradation and the pathogenesis of osteoarthritis. When injury and wear to the cartilage trigger osteoarthritis the down regulation of lubricin is correlated. Interestingly, after early stages of the disease, lubricin expression has increased to levels higher than normal amount. Osteoarthritis is a prevalent disease in America and there are several gaps in the treatment options. With mLub we have looked to replenish lubrication on the joint by binding to the articular surface and reducing friction at the macroscale. We have designed this molecule by starting with a mucin-like chondroitin sulfate backbone with collagen II and hyaluronic acid (HA) binding peptides to promote interaction with the articular surface and synovial fluid constituents.

We have also designed this molecule to resist enzyme degradation from hyaluronidase, MMPs or aggrecanase unlike native molecules in the cartilage and synovial fluid during osteoarthritis. Currently we do not have data for this claim but believe it to be true because the mimic lacks the enzymatically susceptible protein core. Like the previously developed aggrecan mimic and other proteoglycans, we want to see if mLub can also protect other molecules from enzymatic degradation<sup>22</sup>. We have done preliminary studies to find a suitable protocol to detect enzymatic degradation of mLub and the protection by mLub. One possible way to see enzymatic breakdown is with agarose gel electrophoresis. This technique is centered on band separation due to differences in molecular weight. Solutions of cartilage molecules (HA, collagen) and/or mLub are treated with enzymes (hyaluronidase, MMPs or aggrecanase) and then are loaded onto gels with the same

solutions not treated with enzymes. If we see a difference in bands between pairs of untreated and treated, then degradation can be concluded. Otherwise, if we see no difference between bands of mLub alone and mLub treated with an enzyme, then we can conclude mLub is not susceptible to enzymatic degradation. Also, for example if we see a combination of HA or collagen and mLub with no differences of bands after enzyme treatment we can conclude that mLub is protecting the HA from degradation.

Another possible way we have investigated to see the resistance to enzymatic degradation of mLub is by studying the changes of viscosity with a rheometer of solutions before and after an enzyme treatment. The viscosity of HA solutions can be measured with rheology. When HA is degraded into fragments, the solution's dynamic viscosity will decrease. Studying viscosity changes of HA or other cartilage molecule solutions before and after enzyme degradation and with and without mLub protection can be another way to study the protecting ability of mLub.

We have started to work on using both of these methods with just HA, hyaluronidase and mLub and so far our data is inconclusive. This may be due to our protocols, which will need to be revisited before future studies. Another possible reason that we have not seen initial data is that the mLub only contains 5 HA binding peptides and this may not be enough to protect HA. Instead, there are 15 collagen II binding peptides but we have not looked at the protection of this molecule from a collagenase yet. In the future, other molecules and other enzymes should be explored with these two experiments mentioned.

After observing the residency time of mLub of being less than 1 week, further investigation will need to be done with the dosing concentration and timing in vivo. We know that the injection technique of going through the patellar tendon into the synovial area is sufficient and allows the mLub to travel to and bind to the articular cartilage surface. When bound to the surface, the molecule can then act as a lubricant. Currently we only dosed a single 100  $\mu$ L injection of a 0.5 mg/ml mLub solution. Increasing to a higher concentration of the dose, a higher volume or more injections all may need to be studied to find the optimal dosage procedure for best residency time.

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